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Effects of Mutation in *CG11426* Gene on Glial Population in the *Drosophila* Eye Imaginal Disc

초파리의 눈 성충판에서 *CG11426* 유전자 돌연변이가 신경교세포 수에 미치는 영향

2019년 8월

서울대학교 대학원

과학교육과 생물전공

이 종 은

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2019년 7월

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Abstract

Effects of Mutation in *CG11426* Gene on Glial Population in the *Drosophila* Eye Imaginal Disc

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Glial cells have various functions such as guiding axon growth, supporting neuron, taking up neurotransmitters, and forming a blood-brain barrier. However, little is known about the mechanisms for regulating their population. Because *Drosophila* glial cells are highly similar to human glial cells in function and morphology, studying *Drosophila* glial cells is important in understanding the human nervous system. I characterized a novel *Drosophila* gene,

CG11426 which is expressed in glia. *CG11426* encodes lipid phosphate phosphatase (LPP). The known *Drosophila* LPP, Wunens are involved in germ cell migration and survival, and septate junction formation during tracheal development. In this study, I characterized a function of a new gene encoding *Drosophila* LPP, *CG11426*, which regulates glial cell population in the eye imaginal disc. The suppression of the *CG11426* gene increased glial cell number while the ectopic expression of *CG11426* gene reduced glial cell number in the eye imaginal disc. Also, I observed that *CG11426* gene normally promotes apoptosis and inhibits ERK signaling in the eye imaginal disc. Moreover, a mutation in *CG11426* gene caused defects in axon projection to the optic lobe in the larval eye-brain complexes and disruption of brain cortex in adult flies. Thus, the results in this study demonstrate that *CG11426* gene negatively regulates ERK signaling to promote apoptosis and eventually maintains an appropriate glial population.

Keywords: *Drosophila melanogaster*, lipid phosphate phosphatase, glia, apoptosis, ERK signaling, *CG11426*

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Chapter 1. Introduction

1.1. Lipid phosphate phosphatase

Lipid phosphate phosphatase (LPP) is an integral protein with six transmembrane domains and three conserved active sites on the extracellular side (Brindley, 2004; Brindley and Waggoner, 1998; Sigal et al., 2005) (Fig. 1). LPP dephosphorylates lipid phosphates such as lysophosphatidate (LPA) and sphingosine 1-phosphahte (S1P) (Brindley and Waggoner, 1998; Waggoner et al., 1996). These bioactive mediators affect cellular processes such as cell survival, proliferation, differentiation, and migration through G-protein-coupled receptors (GPCRs) in vertebrates (Brindley and Pilquil, 2009; Mendelson et al., 2014; Pyne and Pyne, 2000; Sheng et al., 2015; Tigyi and Parrill, 2003; Yung et al., 2014). For example, insufficient LPA causes vascular developmental failure in mice embryos and yolk sacs (Tanaka et al., 2006; van Meeteren et al., 2006). However, little is known about the *Drosophila* LPP.

So far, Wunen and Wunen2, which are *Drosophila* LPP, have been found and characterized. Wunens regulate germ cell migration and survival (Renault et al., 2010; Starz-Gaiano et al., 2001; Zhang, Zhang et al., 1997). Also, Wunen is required for septate junction during tracheal development (Ile et al., 2012). In this study, I characterized a novel LPP encoding gene called *CG11426*, which is expressed in glia. It was reported that three conserved catalytic

domains in *CG11426* gene are identical to those in human LPP genes (Garcia-Murillas et al., 2006).

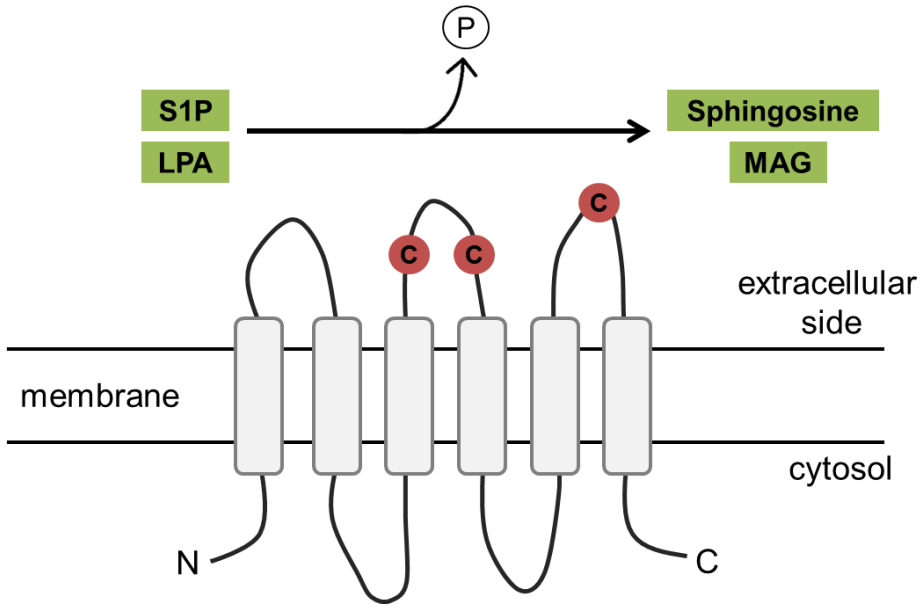

Figure 1. The structure of lipid phosphate phosphatase

CG11426 encodes LPP. LPP has six transmembrane domains (gray) and three conserved active sites (red) on the extracellular side. LPP dephosphorylates lipid phosphates such as lysophosphatidate (LPA) and sphingosine 1-phosphahte (S1P). MAG, monoacylglycerol.

1.2. Glia in the eye imaginal disc

Nervous system consists of neuron and glia. The major cell type (~90%) in our brain is glia, not neuron (Freeman and Doherty, 2006; Stork et al., 2012). Glia have essential roles in the nervous system such as aiding axon growth and pathfinding, protecting, supporting, and maintaining neuron, taking up neurotransmitters, eliminating debris, and forming a blood–brain barrier (Freeman and Doherty, 2006; Hartenstein, 2011; Stork et al., 2012). Although glia have significant functions in the nervous system, their mechanisms for regulating their population are not well explained. In addition, enhanced glial proliferation results in gliomas, the most common human brain tumor (Read et al., 2009; Witte et al., 2009). Therefore, demonstrating the regulation mechanisms of glial cell number is indispensable in order to understand the nervous system.

Because of its morphological and functional similarities to mammalian glia, *Drosophila melanogaster* is an excellent model organism for studying glia (Freeman and Doherty, 2006; Stork et al., 2012). Thus, *Drosophila* can provide potential to significantly improve our understanding of the mammalian glia (Stork et al., 2012). Moreover, *Drosophila* can also provide an opportunity to study glia in the intact organism and powerful molecular tools (Stork et al., 2012). Especially, developing *Drosophila* eye has been used to identify glial cell development because glia in *Drosophila* central nervous system are too complicate to analyze (Choi and

Benzer, 1994; Franzdóttir et al., 2009; Hans et al., 2018; Rangarajan et al., 1999; Reddy and Irvine, 2011, 2013; Silies et al., 2007). Glial cells in the eye disc, called retinal basal glia (RBG), originate from the optic stalk and migrate into the eye disc as photoreceptor (PR) cells differentiate (Choi and Benzer, 1994; Silies et al., 2007). The RBG proliferate during the first and second instar larval stage in the optic stalk and continue proliferating throughout the third instar larval stage both in the optic stalk and the eye disc (Choi and Benzer, 1994; Rangarajan et al., 2001; Silies et al., 2007). Three RBG subtypes are known in the eye disc: perineurial glia (PG), subperineurial glia (SPG), and wrapping glia (WG) (Chotard and Salecker, 2007; Hummel et al., 2002; Silies et al., 2007). The eye disc has two SPG, called carpet glia, which cover the differentiating part of the eye disc (Silies et al., 2007). The PG, located at the basal side of the eye disc, migrate along the carpet glial cells and stop migration when they meet neurons and differentiate into the WG which ensheath axons (Fig. 2A) (Silies et al., 2007). The WG grow their membranes back to the optic stalk and guide axons (Choi and Benzer, 1994; Silies et al., 2007). Glial cells in the eye imaginal disc are essential for appropriate PR axon targeting and pathfinding (Chotard and Salecker, 2007; Rangarajan et al., 1999). *Drosophila* ommatidium consists of eight PR cells; R1–R8 (Ready et al., 1976). The R1–R6 axons terminate in the lamina and form the lamina plexus (Fig. 2B) (Clandinin and Zipursky, 2002). The R7 and R8 axons reach the medulla (Fig. 2B) (Clandinin and Zipursky, 2002). Axons do not enter the optic stalk without glial cells in the eye disc,

indicating that RBG play an important role in guiding PR axons (Rangarajan et al., 1999).

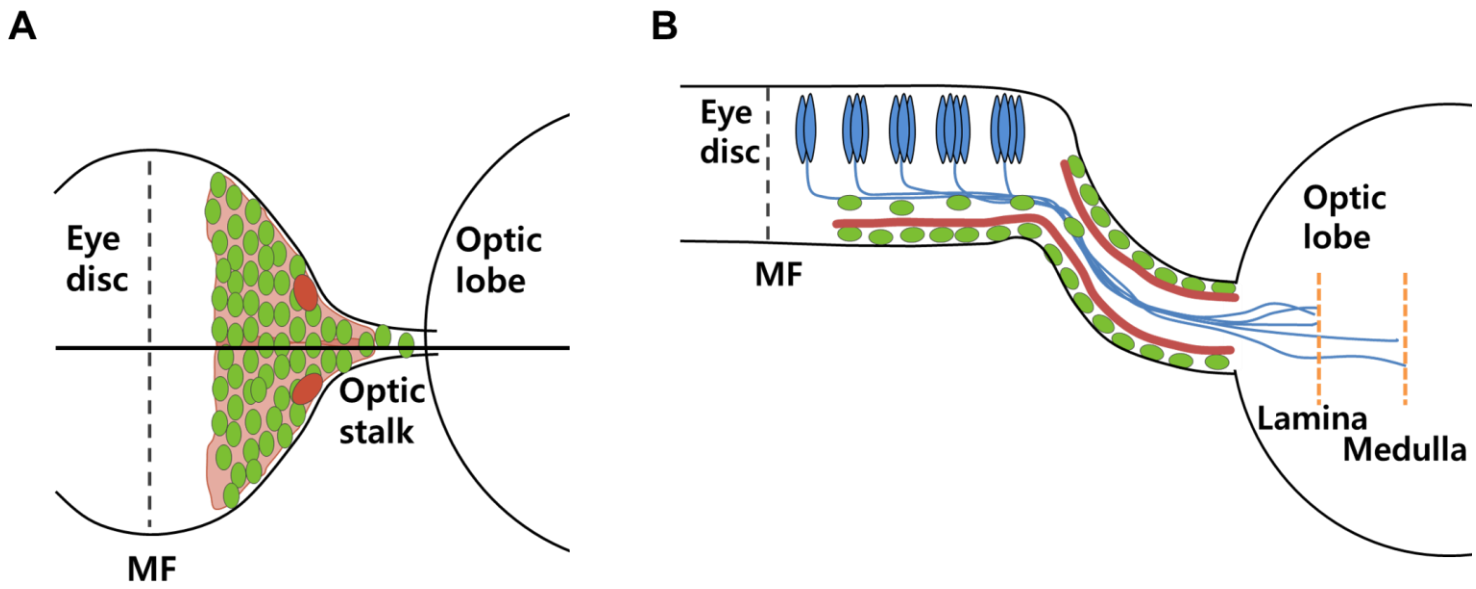

Figure 2. The schematic of eye imaginal disc–brain complex

(A) The schematic of eye imaginal disc–brain complex. Perineurial glia (PG) and wrapping glia (WG) are in green, subperineurial glia (SPG) are in red, and photoreceptor neurons are in blue. The morphogenetic furrow (MF) is indicated by the dashed line. (B) Cross section of the complex shown in A.

1.3. ERK signaling pathway

There have been many reports that changes in cell number are related to signal transduction (Duronio and Xiong, 2013; Green and Llambi, 2015; Raff, 1996). Some previous studies described signaling pathways that affect glial cell number in *Drosophila*; fibroblast growth factor (FGF) signaling controls glia proliferation and migration by Pyramus (Franzdóttir et al., 2009). Epidermal growth factor receptor (EGFR)−Ras and PI3K signaling pathways promote glia proliferation (Read et al., 2009; Witte et al., 2009). Also, EGFR activates Yorkie, a Hippo signaling pathway transcription factor, which promotes cell proliferation (Reddy and Irvine, 2011, 2013), whereas Jun N−terminal kinase (JNK) signaling negatively regulates glial population by reducing Raw (Hans et al., 2018; Luong et al., 2018).

ERK (in *Drosophila*, rolled) belongs to mitogen−activated protein kinase (MAPK) family and regulates activities of specific targets by phosphorylation. Ras activates Raf (in *Drosophila*, pole hole), Raf activates MEK (in *Drosophila*, Dsor1), and finally MEK activates ERK. ERK controls cell proliferation, differentiation, and apoptosis (Huynh et al., 2003; Li et al., 2016; Mebratu and Tesfaigzi, 2009). In *Drosophila*, ERK activates transcription factors such as Pointed (Pnt) and inactivates transcriptional repressors such as Yan (Brunner et al., 1994; O'Neill et al., 1994; Rebay and Rubin, 1995). In a recent study, it was reported that ERK activates Hippo pathway transcription factor Yorkie (Yki) by activating Jub,

Ajuba LIM protein, which binds with Yki kinase Warts and the cofactor Salvador (Reddy and Irvine, 2013). Also Ras/MAPK pathway inhibits cell death by negatively regulating expression of proapoptotic gene *head involution defective* (*hid*) and its activity (Bergmann et al., 1998; Kurada and White, 1998; Sawamoto et al., 1998).

1.4. Purpose of the study

Glia have diverse and important roles in nervous system beyond simply protecting the neurons. Therefore, it is necessary to maintain an adequate number of glia. However, little is known about the regulation of glial population. The purpose of this research is to characterize the functions of a novel LPP gene, *CG11426*, which is expressed in glia. First, the expression pattern of *CG11426* gene was identified. Second, the role of *CG11426* gene in the regulation of glial cell number was examined. Third, the mechanism that the *CG11426* gene regulates glial cell number has been studied. Finally, defects in the nervous system were observed upon mutation in *CG11426* gene. The results in this study show that the new gene, *CG11426*, negatively regulates ERK signaling to promote apoptosis and eventually maintains an adequate glial population, thereby forming an intact nervous system.

Chapter 2. Materials and Methods

2.1. Plasmid construction and *Drosophila* transformation

CG11426 cDNA fragment was amplified from *CG11426* cDNA vector (FI20175, Drosophila Genomics Resources Center (DGRC)) by PCR with the primers 5'-AGTCCTCGAGTAAGCCAGTGAAATGCG-3' and 5'-AGTCTCTAGAAGCATCTTATCCGTCTCAC-3'. So Xho1 site (5'end) and Xba1 site (3'end) were created. The amplified *CG11426* cDNA was cut by Xho1 and Xba1, and inserted between Xho1 and Xba1 sites of pUAST vector, which contains UAS sequences, mini-white marker gene, and p-element ends (Fig. 5A). The construct was analyzed by agarose gel electrophoresis and sequencing. Then the resulting plasmid DNA was injected into w^{1118} embryo (injected by Dr. Kyung-Hee Lee from University of Tennessee). *UAS-CG11426* fly lines were tested using *da-Gal4* (BDSC #55850) whether the *CG11426* is overexpressed (Fig. 5B).

2.2. Fly strains

In this study, these flies were used: *Canton S* (wild type), *CG11426*^{d05846} (Bloominton Drosophila Stock Center (BDSC) #19222), *repo-Gal4* (BDSC #7415), *UAS-CG11426* (I constructed), *UAS-CG11426 RNAi* (BDSC #53879), *CG11426-Gal4* (BDSC #23414), *UAS-GFP.nls* (Kyoto stock center (DGRC) #107870, BDSC #4776), *UAS-mCD8::GFP* (BDSC #32186) and *NP2276-Gal4* (*SPG-Gal4;* DGRC #112853). When it was necessary to distinguish genotypes from larvae, the balancers were replaced.

UAS/Gal4 system was used to knock down the *CG11426* gene in the glia: *UAS-CG11426 RNAi* was crossed to *repo-Gal4*. For overexpression experiment in the glia, *UAS-CG11426* was crossed to *repo-Gal4*. Also, *UAS-CG11426 RNAi* and *UAS-CG11426* were crossed to *NP2276-Gal4*, a *SPG-Gal4* driver. For dpERK staining, *UAS-GFP* (#107870) was induced using *repo-gal4* to define glial cell nuclei. The scheme for fly carrying *UAS-GFP; repo-Gal4/TM3,Sb* is shown in Table 1. Flies were raised at 25℃, 50% humidity and 12 hours light, 12 hours dark condition.

Table 1. The scheme for fly carrying *UAS−GFP; repo−Gal4/TM3, Sb*

P ♂ $w ; \frac{+}{+} ; \frac{repo-Gal4}{TM3,Sb}$ ⓧ $w ; \frac{UAS-GFP.nls}{UAS-GFP.nls} ; \frac{+}{+}$ ♀	P ♂ $w ; \frac{P\{alrm-GAL4.D\}3}{CyO} ; \frac{Dr}{TM3,Sb}$ ⓧ $w^{1118} ; \frac{+}{+} ; \frac{+}{+}$ ♀
F1 ♂ $w ; \frac{UAS-GFP.nls}{+} ; \frac{repo-Gal4}{+}$	F1 ♀ $w ; \frac{CyO}{+} ; \frac{TM3,Sb}{+}$

P ♂ $w ; \frac{UAS-GFP.nls}{+} ; \frac{repo-Gal4}{+}$ ⓧ $w ; \frac{CyO}{+} ; \frac{TM3,Sb}{+}$ ♀

F1 $w ; \frac{UAS-GFP.nls}{CyO} ; \frac{repo-Gal4}{TM3,Sb}$

P ♂ $w ; \frac{UAS-GFP.nls}{CyO} ; \frac{repo-Gal4}{TM3,Sb}$ ⓧ $w ; \frac{UAS-GFP.nls}{CyO} ; \frac{repo-Gal4}{TM3,Sb}$ ♀

F1 $w ; \frac{UAS-GFP.nls}{UAS-GFP.nls} ; \frac{repo-Gal4}{TM3,Sb}$

2.3. Immunohistochemistry

The third Instar larvae were dissected in 1x PBS with 0.1% Triton (PBST), fixed in 4% paraformaldehyde for 20 minutes, washed, and blocked in PBST with 5% normal goat serum (Wu and Luo, 2006). For dpERK staining, the third instar larvae were dissected in 8% paraformaldehyde with phosphatase inhibitor cocktail (GenDEPOT), washed, and permeabilized with 0.5% PBST. The next step is the same as described above.

Embryos were collected and dechorionated using 50% bleach for 5 minutes. After washing, the fixation was performed in 1:1 mixture of heptane and 4% paraformaldehyde for 25 minutes on the shaker. After paraformaldehyde was removed, an equal volume of cold methanol was added and shaken for 25 minutes. Heptane was removed and 3ml cold methanol was added. The samples were shaken vigorously and washed with 0.1% PBST.

The used antibodies are as follows: mouse anti-Repo (1:20, Developmental Studies Hybridoma Bank (DSHB)), rabbit anti-GFP (1:1000, Invitrogen), mouse anti-MAP Kinase, activated (anti-dpERK; 1:1000, Sigma-Aldrich), rabbit anti-Dcp-1 (1:100, Cell Signaling Technology), mouse 24B10 (anti-chaoptin; 1:100, DSHB), and Alexa Fluor 594 conjugated anti Horseradish Peroxidase (HRP) (1:500, Invitrogen). The used secondary antibodies are as follows: Alexa Fluor 488 anti-mouse (1:1000, Invitrogen), Alexa Fluor 594 anti-mouse (1:1000, Invitrogen), and

Alexa Fluor 488 anti-rabbit (1:1000, Invitrogen).

Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) was used for ethynyl deoxyuridine (EdU) labeling to detect S-phase cells (Daul et al., 2010). The third Instar larvae were dissected in Schneider's *Drosophila* Medium (Gibco), incubated in Schneider's *Drosophila* Medium with 20 μM EdU for 10 minutes, and fixed in 4% paraformaldehyde for 25 minutes. Samples were washed, permeabilized with 0.5% PBST for 5 minutes, washed, and blocked in PBST with 5% normal goat serum. The Click-iT reaction was carried out as the manufacturer's instruction. After washing, immunostaining was performed as described above.

For 24B10 antibody staining, eye-brain complexes of the third instar larvae were dissected and stained with 24B10 antibody and ImmPRESS HRP Anti-Mouse IgG (Peroxidase) Polymer Detection Kit, made in Horse (Vector Laboratories). Then the eye-brain complexes were incubated in DAB with 3.4% H_2O_2.

The fluorescent images were obtained using LSM 780 confocal microscope, and the bright field images were obtained using Olympus BX51 microscope. The number of glia was counted manually in the eye disc with 17-20 rows of photoreceptor neurons. The intensity of dpERK was quantitated by measuring the mean intensity of the glia region in the eye imaginal disc, and this was normalized to the mean intensity of the anterior area to the morphogenetic furrow. For Dcp-1 staining, percentage of area with Dcp-1 staining in the total area of the eye disc was quantitated (Meserve and Duronio, 2015). These were analyzed using Image J.

2.4. Histology

Heads of 1 day aged adult flies were fixed in Carnoy's solution (100% EtOH : chloroform : glacial acetic acid = 6:3:1) for 4 hours, dehydrated in 95% EtOH twice for 30 minutes each, 100% EtOH for 1 hours, and incubated in xylene overnight. The heads were incubated in a 1:1 mixture of xylene and paraffin twice at 85℃ for 30 minutes and in paraffin for five times at 85℃ for 30 minutes. The heads were embedded in the paraffin, sectioned to 5㎛ (Sunderhaus and Kretzschmar, 2016). Then the samples were stained with hematoxylin and eosin. The images were obtained using Zeiss Axio Imager A1 microscope.

2.5. Quantitative Real-time PCR (qRT-PCR)

1 day aged adult flies were collected and frozen at -80℃. Total RNA extraction was performed according to RNA isolation kit (MACHEREY-NAGEL). The eluted RNA was reverse transcribed using 5X all-in-one RT master mix (abm). qRT-PCR was performed with the synthesized cDNA using TOPreal qPCR 2X premix (SYBR Green with low ROX; enzynomics) and Roter-gene Q instrument (Qiagen). The following primers were used: for *RpLP* 5'-TAATCTCGGCAGTTTGAACG-3' and 5'-CACGCTGGTCAAAATCCTAA-3'; for *CG11426* 5'-

GTCCGTAACGGTTTCCATGC-3' and 5'-ACGCAGATGGGAATGACGAG-3'. Each sample was normalized to *RpLp*.

Chapter 3. Results

3.1. *CG11426* gene was expressed in glia at embryonic and larval stage

To examine the *CG11426* gene expression, *CG11426* enhancer trap line (*CG11426-Gal4*) and *UAS-green fluorescent protein (GFP)* were used (*CG11426* antibody was not available). *CG11426-Gal4* was crossed to *UAS-GFP.nls* (#4776) which is tagged with nuclear localization signal at N terminal end. *CG11426>GFP* larval brains and eye imaginal discs were dissected and stained with anti-GFP and anti-Repo antibodies (Fig. 3A, C). The GFP expression overlapped with that of reversed polarity (Repo), a glia-specific marker, in both optic lobe and eye disc. In the eye imaginal disc, it was particularly expressed in carpet glia (Fig. 3A). *Drosophila* eye imaginal disc has two carpet glia; one covers half of the differentiating part of the disc and the other covers the remaining half (Silies et al., 2007). *CG11426-Gal4* was then crossed to *UAS-mCD8::GFP* to analyze the membrane of the *CG11426* expressing cells. Likewise, *CG11426>mCD8::GFP* eye imaginal discs were dissected and stained. I confirmed that the differentiating part of the disc was covered with the cells expressing *CG11426* (Fig. 3B). This result shows that *CG11426* is expressed in the carpet glia of the eye imaginal disc. *CG11426* and

repo expression also overlapped in *CG11426>GFP* embryos (Fig. 4). These observations indicate that *CG11426* is expressed in the glia.

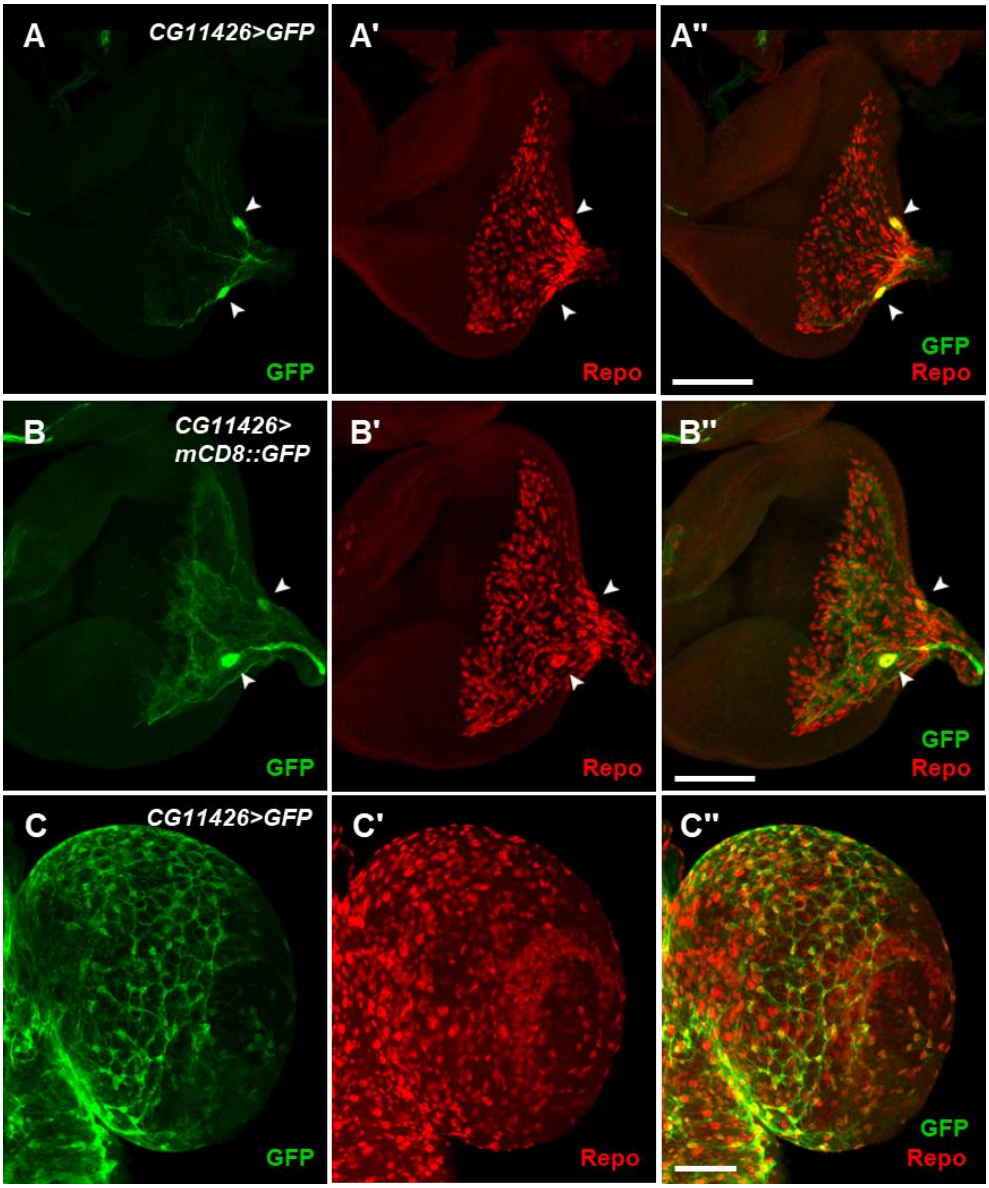

Figure 3. *CG11426* expression in the larval eye imaginal discs and optic lobe

(A–A'', C–C'') *CG11426* expression (GFP; green) and glial nuclei (anti–Repo, glia–specific marker; red) in *CG11426>GFP* larval eye imaginal disc (A–A''), and larval optic lobe (C–C''). The *CG11426* expression overlapped with *repo* expression in both the eye imaginal disc and the optic lobe (yellow in A'', C''). **(B–B'')** A

membrane of *CG11426* expressing cell (GFP; green) and glial nuclei (anti-Repo; red). *CG11426* is expressed in carpet glia (arrowheads) covering the area with RBG (yellow in A'', B''). A–C' show a single channel. A''–C'' show the merged image. Posterior to the right (A– B''). Scale bar, 50 μm.

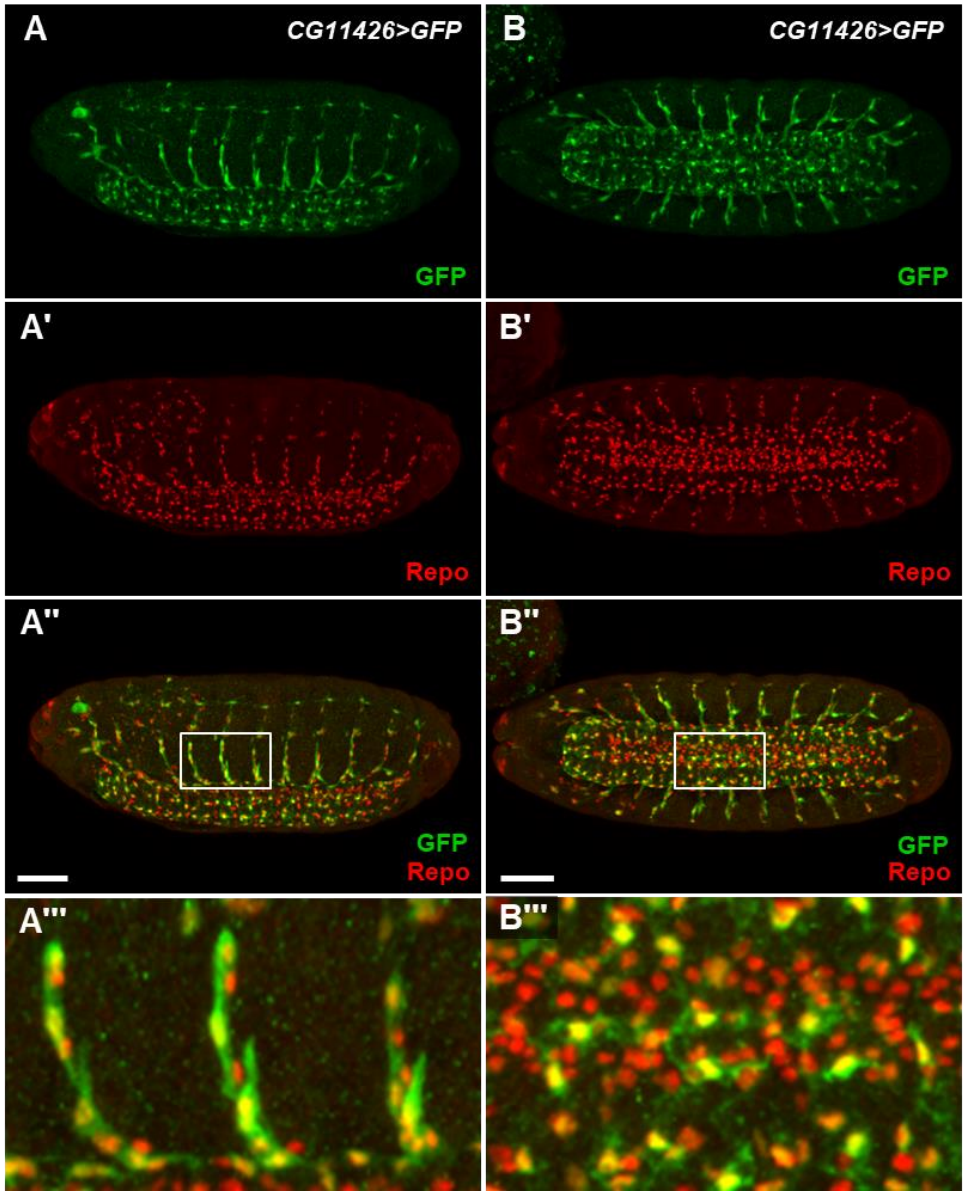

Figure 4. *CG11426* expression in the embryo

(A–B'') *CG11426* expression (GFP; green) and glial nuclei (anti-Repo; red) in *CG11426>GFP* embryos. Posterior to the right. *CG11426* is expressed in glia of the embryo (yellow in A'', B''). (A–A'') Lateral view. (B–B'') Ventral view. A–B' show a single channel. A''–B'' show the merged image. **(A''', B''')** Box area from A'' and B''. Scale bar, 50 μm.

3.2. *UAS-CG11426* flies were constructed

To overexpress the *CG11426* gene, *UAS-CG11426* fly lines were produced (Fig. 5). Four independent lines, 1A, 2A, 3A, and 5B, were assessed. All three lines except 5B, have *UAS-CG11426* construct on the third chromosome while 5B has the construct on the second chromosome.

The efficacy of the *UAS-CG1426* lines was tested by qRT-PCR. Each fly line was crossed with *da-Gal4*, a ubiquitous driver. The relative expression levels were confirmed: expression of *CG11426* gene was about 82 fold higher in *da>CG11426 (1A)* flies, 66 fold higher in *da>CG11426 (2A)* flies, 86 fold higher in *da>CG11426 (3A)* flies, and 113 fold higher in *da>CG11426 (5B)* flies than in *da-Gal4* control flies (Fig. 5B). This indicates that constructed *UAS-CG11426* lines work well. Among these lines, 3A line was mainly used in this paper. Using the *UAS-CG11426 (3A)* flies, the gain of function phenotypes of *CG11426* were analyzed.

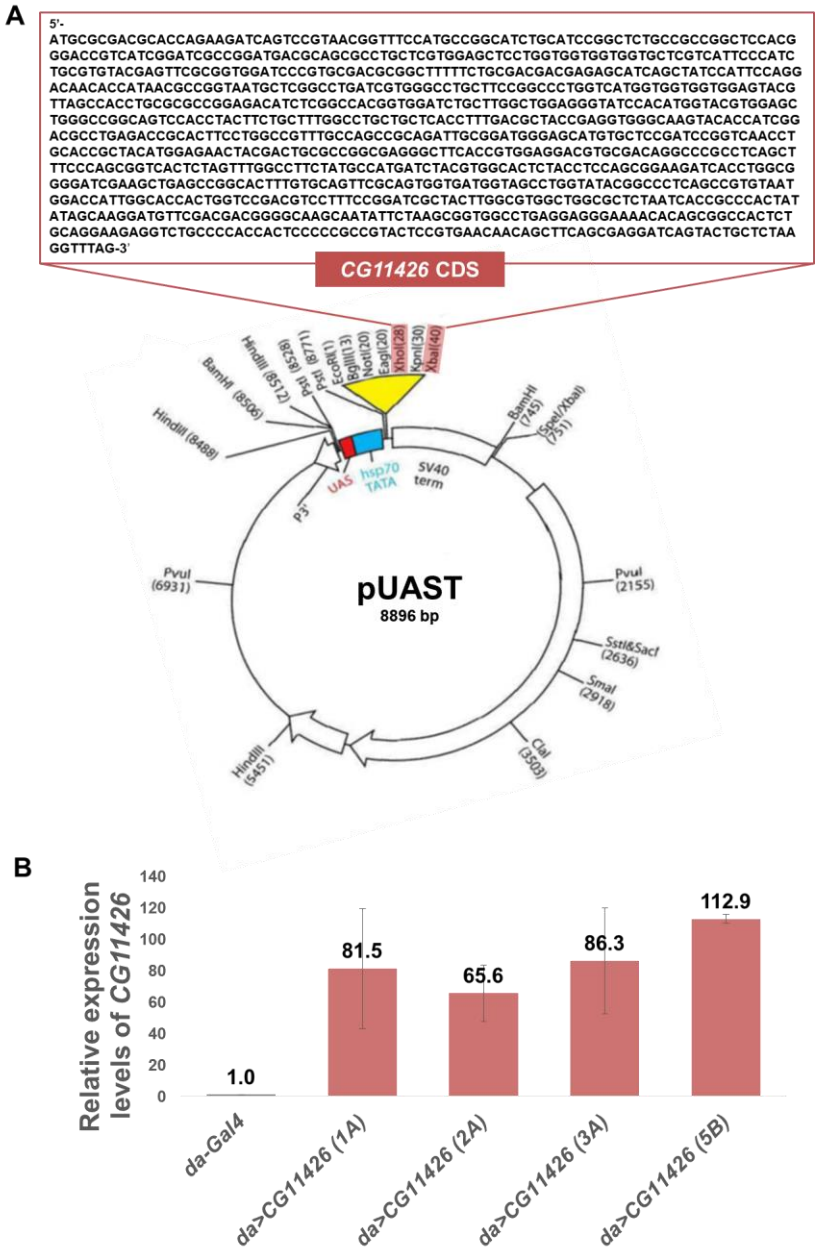

Figure 5. Construction of *UAS-CG11426* fly lines

(A) pUAST plasmid map and the coding sequence of *CG11426* gene. *CG11426* cDNA was inserted between Xho1 and Xba1 sites of the pUAST vector. (B) *CG11426* expression levels in the constructed *UAS-CG11426* flies; 1A, 2A, 3A, 5B. A ubiquitous driver, *da-Gal4,* was used to drive the *CG11426* gene.

3.3. *CG11426* gene negatively regulated the number of glial cells in the eye imaginal disc

I then investigated how the mutation of *CG11426* gene affects glia. The third instar larval eye imaginal discs with 17−20 rows of photoreceptor neurons (defined by staining with HRP) were dissected and stained with Repo antibody. Since the *CG11426* homozygous mutants die before the third instar larval stage, the *CG11426* heterozygous mutants ($CG11426^{19222/+}$) were analyzed. The eye imaginal discs of the *CG11426* mutant had an average of 231 glial cells (Fig. 6B, C) whereas those of wild type had 194 glial cells on average (Fig. 6A, C). The number of glial cells in the *CG11426* mutants significantly increased compared to that of wild type. To investigate the role of the *CG11426* gene, especially in glia, *CG11426* was knocked down in glia (*repo>CG11426 RNAi*) using RNA interference (RNAi) and overexpressed in glia (*repo>CG11426*) using the *UAS−CG11426* line. While the eye imaginal discs of *repo−Gal4* control had 187 glial cells on average (Fig. 7A, D), those of *repo>CG11426 RNAi* flies had an average of 237 glial cells (Fig. 7B, D). Like the $CG11426^{19222}$ mutants, *CG11426* knockdown in glia considerably increased the number of glial cells. On the other hand, *repo>CG11426* eye imaginal discs had an average of 147 glial cells (Fig. 7C, D), which was a significant decrease in glial cells. These observations suggest that *CG11426* negatively regulates the number of glial cells in the eye imaginal

disc. In other words, it means that *CG11426* normally functions in maintaining the appropriate number of glia. In addition, *repo>CG11426* eye imaginal discs were smaller than those of control (Fig. 7C). This suggests that reduced number of glial cells due to *CG11426* overexpression leads to poor development of the eye disc.

3.4. *CG11426* expression levels were identified in the mutant flies

To make sure that *CG11426* is well knocked down or overexpressed, quantitative real-time PCR analysis was performed. *repo>CG11426* showed higher expression of *CG11426* than *repo-gal4* controls, and *repo>CG11426 RNAi* showed lower expression of *CG11426* than the controls (Fig. 7E). *CG11426* heterozygous mutants had lower expression of *CG11426* compared to wild type flies (Fig. 6D).

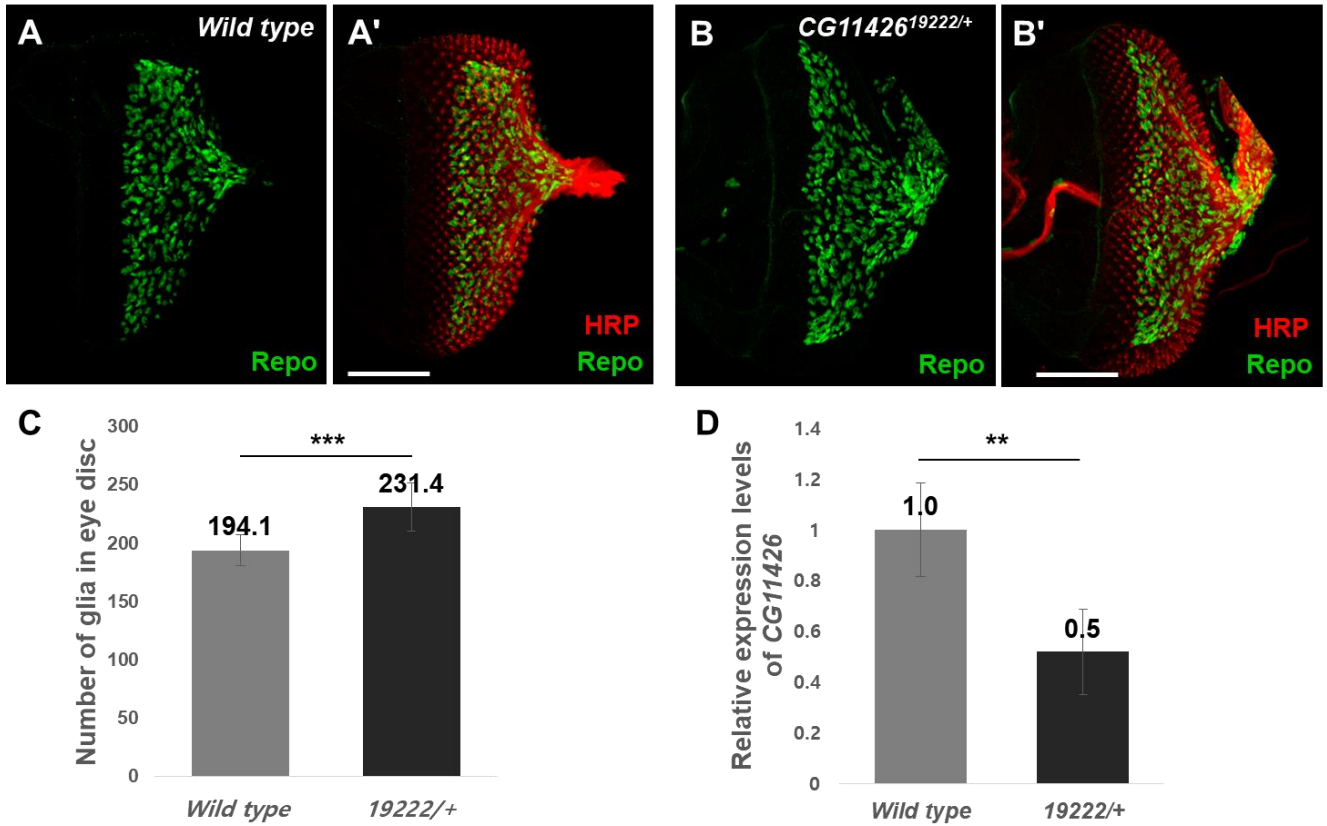

Figure 6. Glial cell number in the eye imaginal discs of *CG11426*$^{19222/+}$

(A–B') Glial staining (anti–Repo; green) and photoreceptor staining (anti–HRP; red) of eye imaginal discs.

Posterior to the right. (A, A') Wild type(n=8). (B, B') *CG11426* mutant had increased glial cell number (*CG11426*$^{19222/+}$; n=8). A–B show a single channel. A'–B' show the merged image. **(C)** Quantitation of the number of glial cells in the eye imaginal disc; A–B. **(D)** Quantitation of the relative *CG11426* expression levels in the flies; A–B. Significance was determined by Student's t–test, **p<0.01, ***p<0.001. Scale bar, 50 μm.

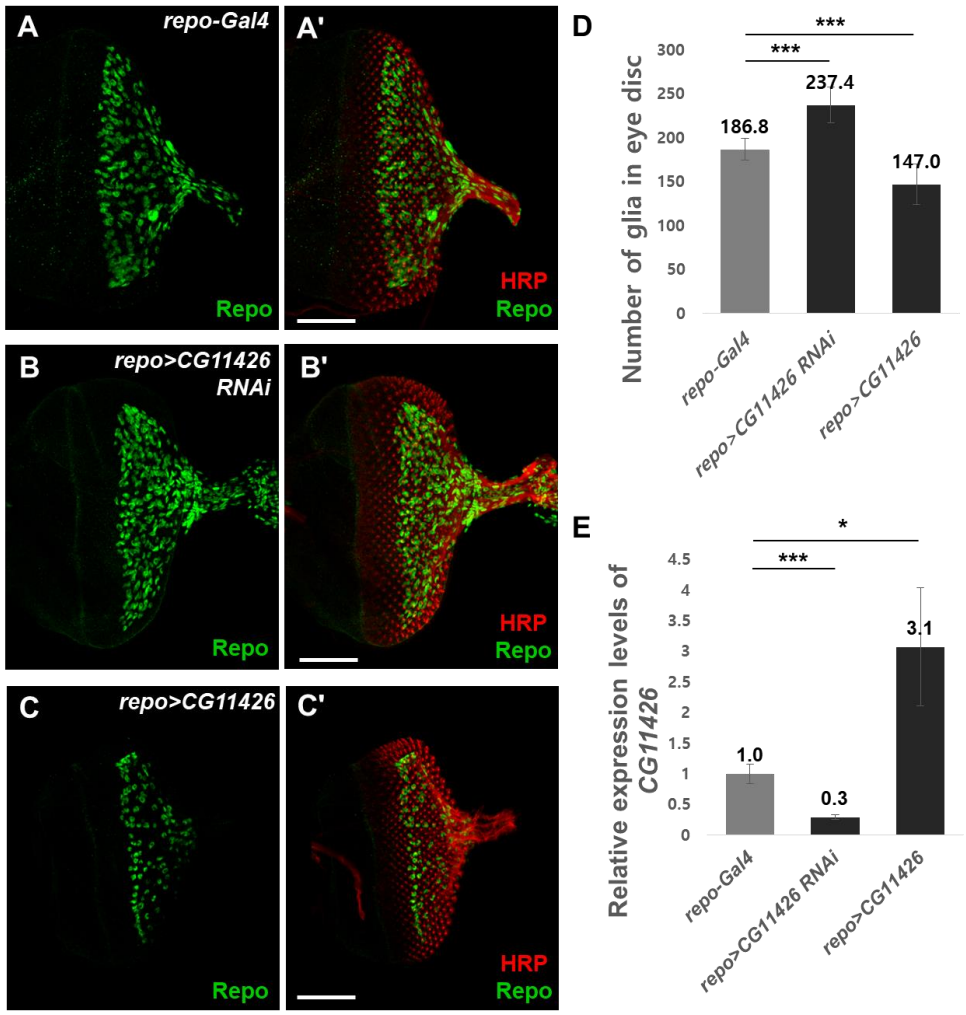

Figure 7. Glial cell number in the eye imaginal discs of *repo>CG11426 RNAi* and *repo>CG11426*

(A–C') Glial staining (anti–Repo; green) and photoreceptor staining (anti–HRP; red) of eye imaginal discs. Posterior to the right. (A, A') Control animals (*repo–Gal4*; n=9). (B, B') *CG11426* knockdown in glia increased glial population (*repo>CG11426 RNAi*; n=9). (C, C') *CG11426* overexpression in glia decreased glial population (*repo>CG11426*; n=9). A–C show a single channel. A'–C' show the merged image. **(D)** Quantitation of the number of glial cells in the

eye imaginal disc; A–C. **(E)** Quantitation of the relative *CG11426* expression levels in the flies; A–C. Significance was determined by Student's t–test, *p<0.05, ***p<0.001. Scale bar, 50 μm.

3.5. *CG11426* knockdown in the SPG decreased the number of glial cells

Since *CG11426* was specifically expressed in carpet glia, I observed the requirements for *CG11426* in carpet glia. To knock down or overexpress *CG11426* in the SPG, *SPG-Gal4* was used. *CG11426* knockdown in the SPG (*SPG>CG11426 RNAi*) increased the number of glia (214 glial cells; Fig. 8B, D) compared to *SPG-Gal4* controls (196 glial cells; Fig. 8A, D). This was less severe phenotype than *repo>CG1126 RNAi*. *CG11426* overexpression in the SPG (*SPG>CG11426*) did not show a significant change in the number of glial cells (197 glial cells; Fig. 8C, D) compared to controls. These results are expected because *CG11426* would have been less knocked down or overexpressed by *SPG-Gal4* driving than *repo-Gal4* driving. So it would not have been enough to appear as a strong phenotype. *CG11426* is LPP which dephosphorylates extracellular LPA and S1P. Therefore, the concentration of extracellular LPA and S1P varies depending on how much LPP is expressed. The gene was expressed only in a specific glia by *SPG-Gal4* driving, while it was expressed in most glia by *repo-Gal4* driving. *UAS-GFP.nls* (#4776) flies were crossed to *SPG-Gal4* flies to confirm the *SPG-Gal4* expression. This confirmed that *SPG-Gal4* is expressed in the SPG of the eye imaginal disc (Fig. 9). However, sometimes *SPG-gal4* expression was observed in only one disc out of two or in only one carpet glia out of two. In

addition, *SPG-Gal4* driver was expressed at a later embryonic stage than Repo (data not shown). Nevertheless, these results show that *CG11426* has vital roles in glia, especially in carpet glia. In addition to controlling glial migration, this study found a novel function of the carpet glia that regulates the population of glial cells by the *CG11426* gene in the eye imaginal disc.

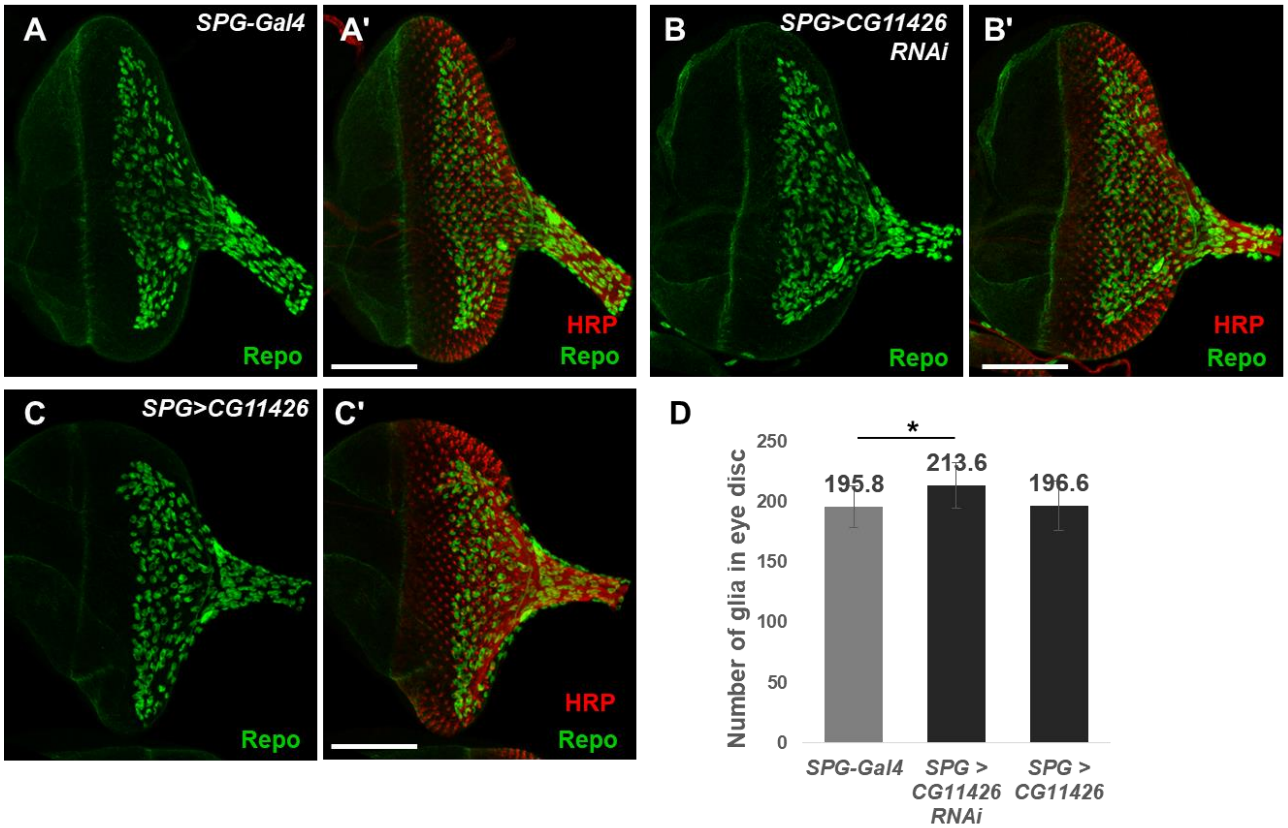

Figure 8. *CG11426* knockdown in subperineurial glia

(A–C') Glial staining (anti–Repo; green) and photoreceptor staining (anti–HRP; red) of eye imaginal discs.

Posterior to the right. (A, A') Control animals (*SPG-Gal4*; n=11). (B, B') *CG11426* knockdown in SPG mildly increased glial population (*SPG>CG11426 RNAi*; n=10). (C, C') *CG11426* overexpression in SPG had no change in glial population (*SPG>CG11426*; n=11). A-C show a single channel. A'-C' show the merged image. **(D)** Quantitation of the number of glial cells in the eye imaginal disc; A-C. Significance was determined by Student's t-test, *p<0.05. Scale bar, 50 μm.

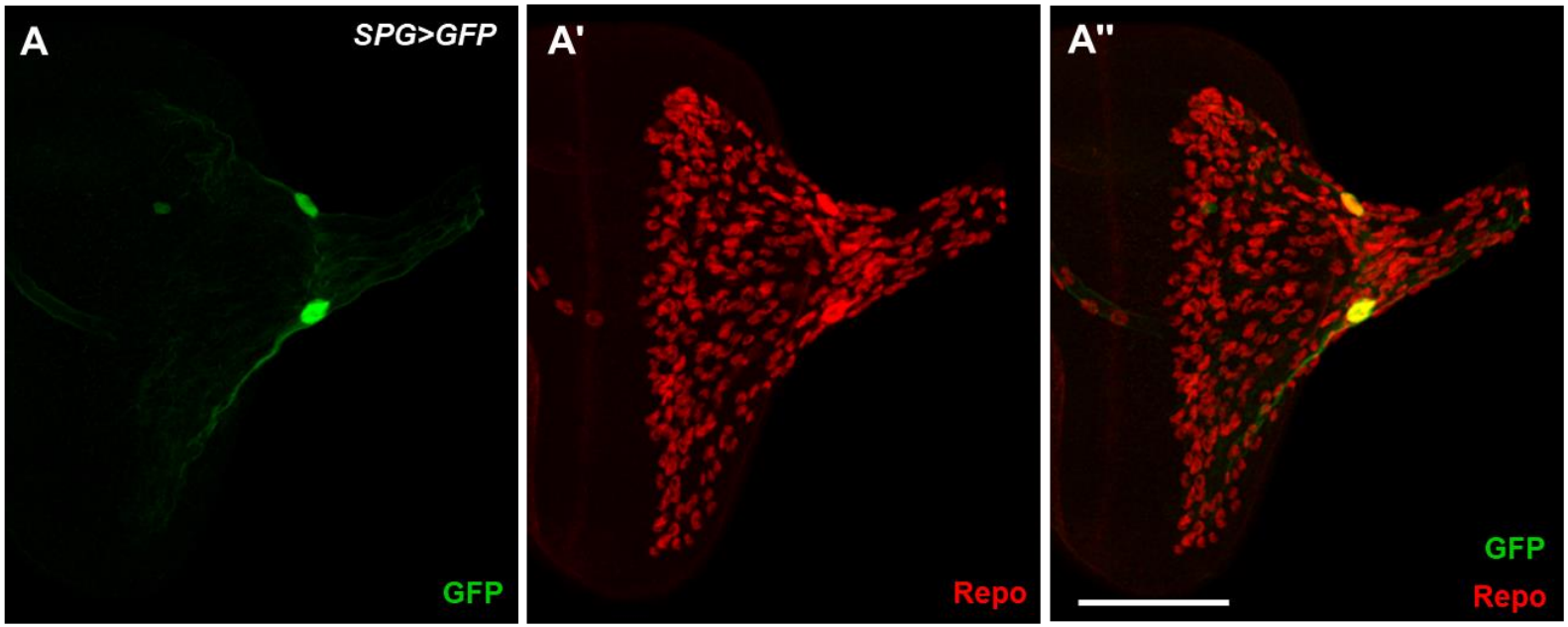

Figure 9. *SPG-Gal4* expression in the eye imaginal disc

SPG-Gal4 expression (GFP; green) and glial nuclei (anti-Repo; red) in the *SPG>GFP* eye imaginal disc. A, A' show a single channel. A'' shows the merged image. Scale bar, 50 μm.

3.6. *CG11426* gene promoted glial cell death

Cell number is regulated by a balance between cell death and cell proliferation. So, I tried to find out which affects glial cell number in the *CG11426* mutants. The eye imaginal discs were stained with cleaved death caspase (Dcp-1) antibody to determine if the changes in glial cell number are associated with cell death. The area with Dcp-1 staining was reduced in *repo>CG11426 RNAi* (average 0.94% of the area in the eye disc; Fig. 10B, D) and it was increased in *repo>CG11426* (average 2.47% of the area in the eye disc; Fig. 10C, D) compared to *repo-Gal4* controls (average 1.64% of the area in the eye disc; Fig. 10A, D). These observations supported that *CG11426* is associated with an increase in glial cell death.

Then, cell proliferation was examined in *CG11426* mutants. To determine whether the changes in glial cell number are an effect of cell proliferation, ethynyl deoxyuridine (EdU) was used to label S-phase cells. EdU labeling showed that there is no difference in the percentage of glial cells in S-phase between the controls and the *CG11426* mutants (Fig. 11). These results show that *CG11426* regulates glial cell number in the eye imaginal disc by apoptosis, but not by proliferation. In other words, *CG11426* normally inhibits excessive glial cell number and maintains an adequate number of glial cells by apoptosis in the eye imaginal disc.

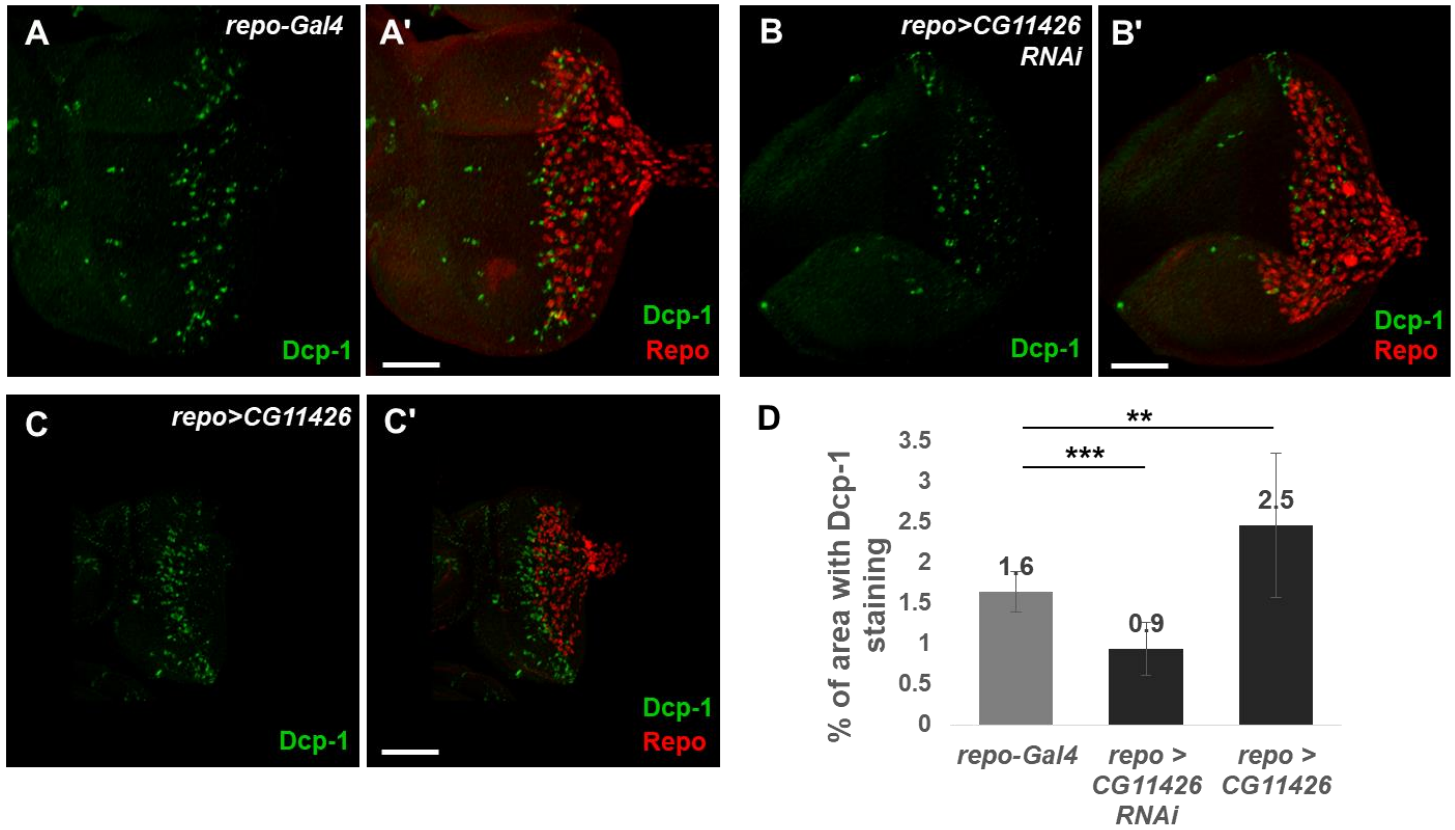

Figure 10. *CG11426* promoted apoptosis in glia

(A–C') Cell death staining (anti-Dcp-1; green) and glial staining (anti-Repo; red) of eye imaginal discs.

Posterior to the right. (A, A') Control animals (*repo-Gal4*; n=14). (B, B') *CG11426* knockdown in glia reduced apoptosis (*repo>CG11426 RNAi*; n=15). (C, C') *CG11426* overexpression in glia increased apoptosis (*repo>CG11426*; n=13). (D) Quantitation of percentage of total area with Dcp-1 staining in the eye imaginal disc; A-C. Significance was determined by Student's t-test, **p<0.01, ***p<0.001. A-C show a single channel. A'-C' show the merged image. Scale bar, 50 μm.

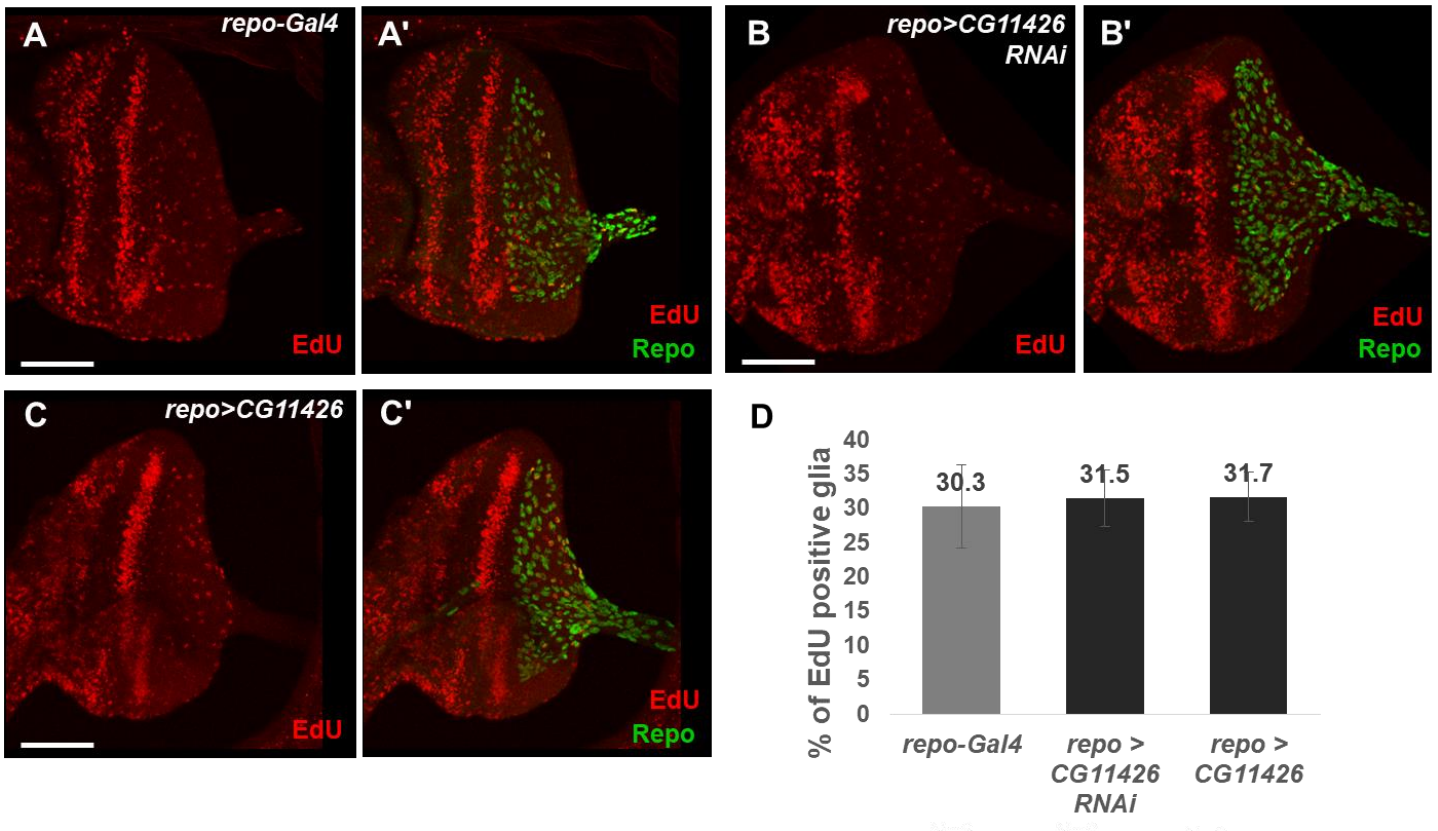

Figure 11. *CG11426* mutation had no effect on proliferation

(A–C') Proliferating cells marked with EdU (red) and glial cells marked with Repo (green) in the eye imaginal

disc. Posterior to the right. (A) Control animals (*repo-Gal4*; n=6). (B) *CG11426* knockdown in glia (*repo>CG11426 RNAi*; n=9). (C) *CG11426* overexpression in glia (*repo>CG11426*; n=9). (D) Quantitation of percentage of glial cells in S-phase in the eye imaginal disc; A-C'. Significance was determined by Student's t-test. No significant differences were observed between A, B and C. A-C show a single channel. A'-C' show the merged image. Scale bar, 50 μm.

3.7. *CG11426* gene suppressed ERK signaling

As the control of cell number is in general related to signaling pathway, I tried to find out which signaling is associated with regulation of cell number by the *CG11426* gene product. First, I searched for the signaling pathways known to be associated with LPP and searched mutants that showed similar phenotypes to those seen in the *CG11426* gene mutation. ERK signaling was selected as a candidate, considering that the ERK mutant showed a similar phenotype to the *CG11426* mutant and that LPA and S1P activate ERK signaling in mammalian cells (Castillo and Teegarden, 2003; Franzdóttir et al., 2009; Kim et al., 2003; Reddy and Irvine, 2013; Sui et al., 2015; Wang et al., 2015). I found that *CG11426* regulates the number of glial cells through ERK signaling pathway. Reduced *CG11426* levels increased dually phosphorylated ERK (dpERK; activated ERK) levels and conversely, increased *CG11426* levels decreased dpERK levels compared to controls (Fig. 12A–D). These results support that the *CG11426* gene negatively regulates glial population in the *Drosophila* eye imaginal disc by inhibiting ERK signaling pathway.

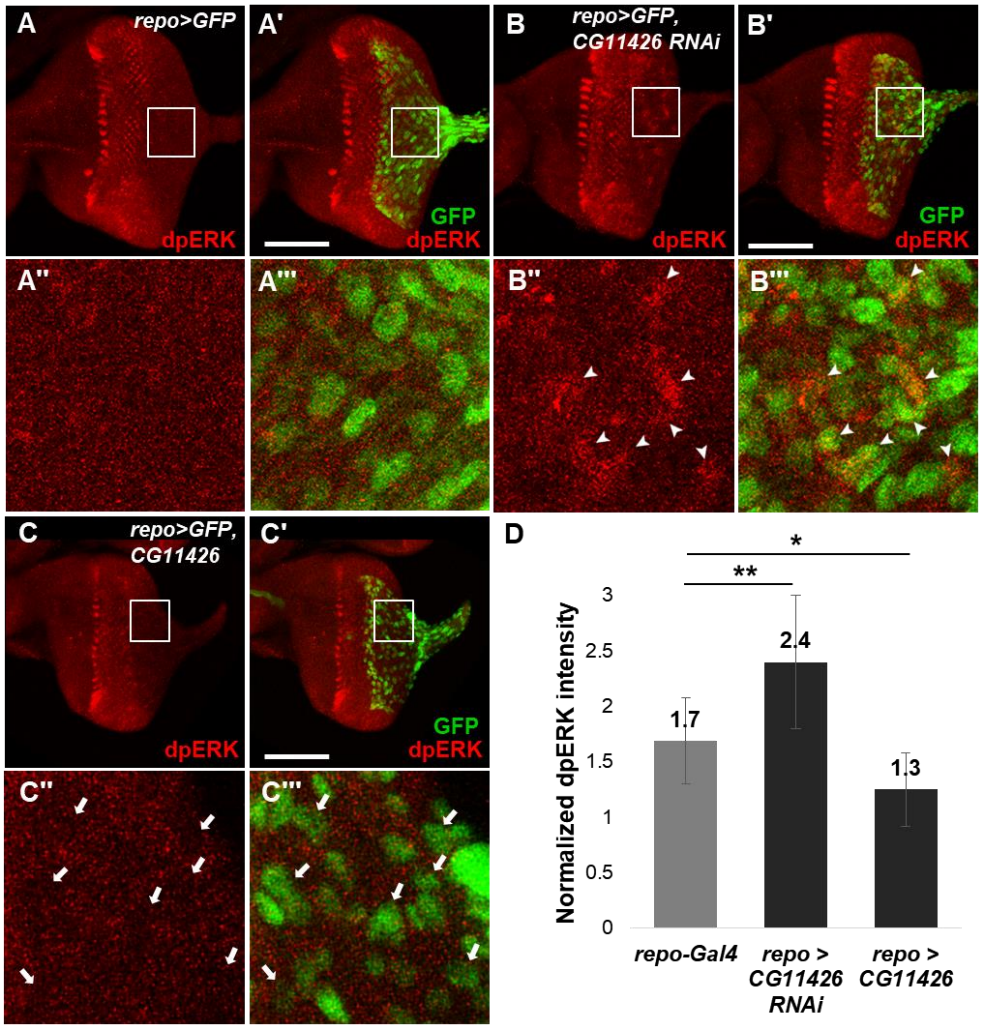

Figure 12. *CG11426* suppressed ERK signaling

(A–C') Activated-ERK expression (anti-dpERK; red) and glial nuclei (GFP; green) of eye imaginal discs. Posterior to the right. (A, A') Control animals (*repo>GFP*; n=7). (B, B') *CG11426* knockdown in glia increased dpERK levels (*repo>GFP, CG11426 RNAi*; n=11). (C, C') *CG11426* overexpression in glia decreased dpERK levels (*repo>GFP, CG11426*; n=8). A–C show a single channel. A'–C' show the merged image. **(A''–C''')** Box area from A–C'. The arrowheads indicate increased dpERK levels. The arrows indicate

decreased dpERK levels. (D) Quantitation of average dpERK intensity in glia region relative to anterior region of the eye imaginal disc. Significance was determined by Student's t-test, *$p<0.05$, **$p<0.01$. Scale bar, 50 μm.

3.8. Mutation in *CG11426* gene caused defects in axon projection

Glial cells in the eye imaginal disc are essential for appropriate photoreceptor (PR) axon targeting and pathfinding (Chotard and Salecker, 2007; Rangarajan et al., 1999). Previous studies have shown that the decrease in the number of WG results in abnormalities in the PR axon projection, such as more and larger holes in the lamina (Chang et al., 2018). To test whether there are problems with axon projection in the *CG11426* mutants, the third instar larval eye-brain complexes were stained with 24B10 antibody, a PR marker. Similarly, more and larger holes were observed in the lamina of *repo>CG11426* with decreased glial cell number (Fig. 13C). Plus, R7-R8 axons did not extend widely to the medulla and optic lobes were smaller than those of control (Fig. 13C). These suggest that a reduced number of glial cells due to *CG11426* overexpression causes defects in the development of the brain. Holes were also observed in *repo>CG11426 RNAi* with increased glial cell number (Fig. 13B). Also, lamina plexus was uneven (Fig. 13B). Moreover, holes were often observed in the medulla (data not shown). These show that increased glial cell number by *CG11426* knockdown also causes holes, which suggests defects in axon projetion.

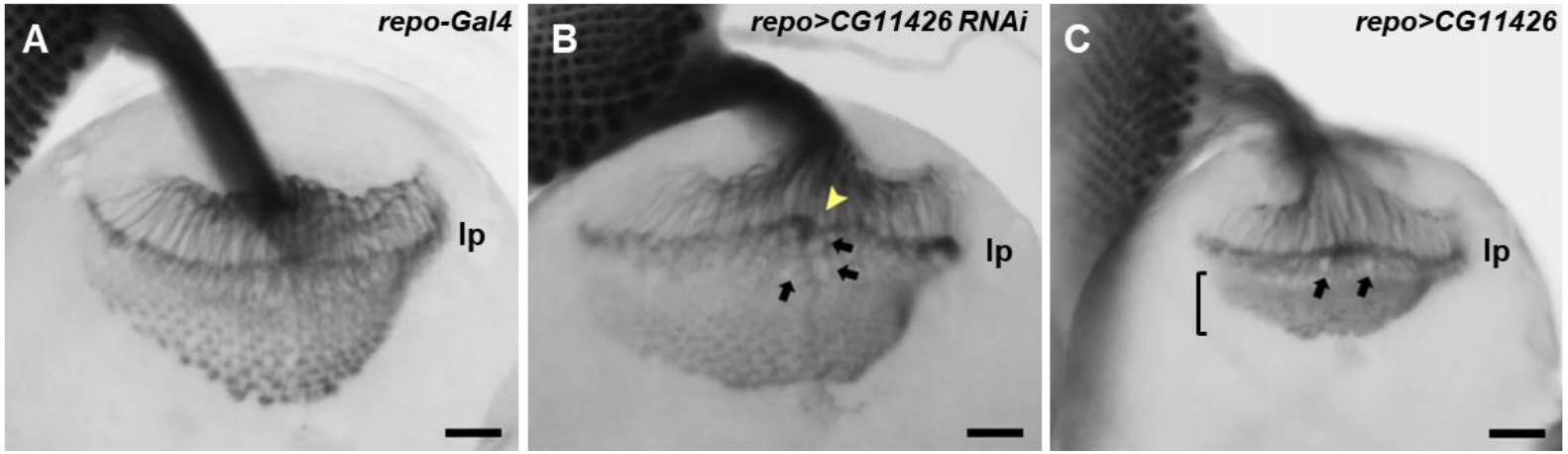

Figure 13. Mutation in *CG11426* gene caused defects in axon projection

R cell axons were marked with 24B10 antibody in larval eye-brain complexes. (A) Control animals (*repo-Gal4*). (B) *CG11426* knockdown in glia caused holes in lamina plexus (lp) (arrows), and the lamina plexus was uneven (arrowhead) (*repo>CG11426 RNAi*). (C) *CG11426* overexpression in glia caused holes in lamina plexus (arrows), and the R7/R8 axon projection was abnormal (bracket) (*repo>CG11426*). Scale bar, 20 μm.

3.9. Increased *CG11426* levels caused defects in integrity of brain

I hypothesized that since the glia play important roles in the nervous system, abnormal number of glia will lead to an abnormal nervous system. To examine the effect of *CG11426* mutation in adult brains, paraffin histology and H&E staining were performed. Severe neuropathology was observed in *repo>CG11426* brains. The nuclei in the brain cortex were lost (Fig. 14C). However, there was no significant change in *repo>CG11426 RNAi* brains compared to the *repo-Gal4* controls (Fig. 14A, B).

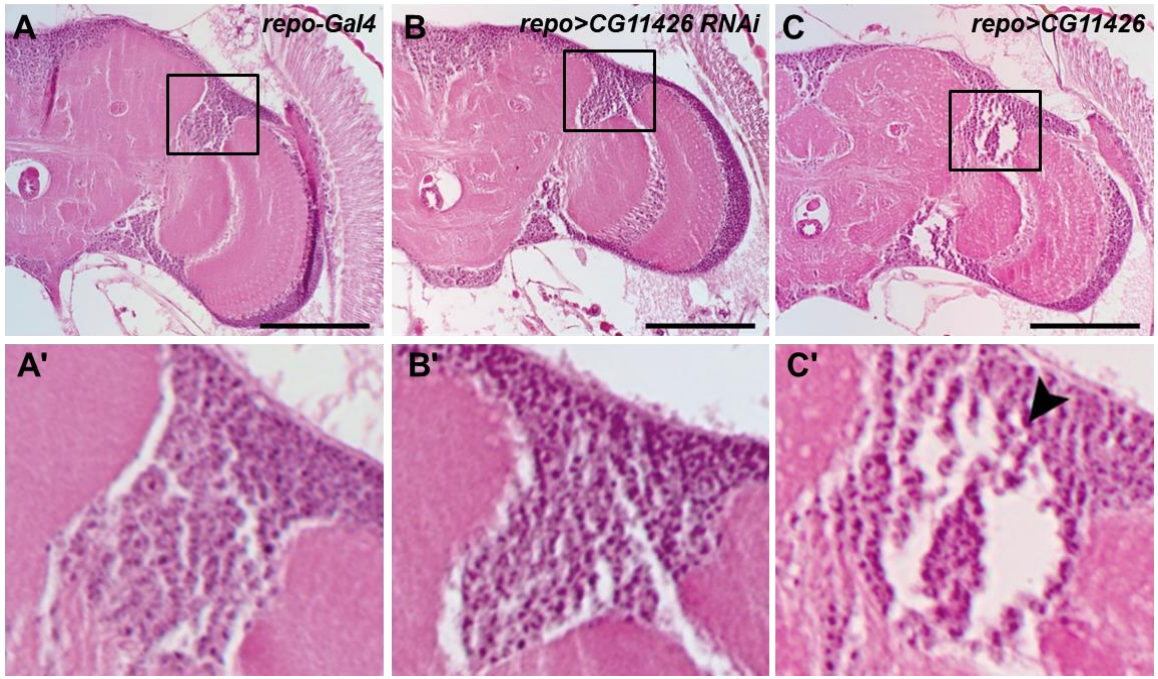

Figure 14. Increased *CG11426* levels caused defects in integrity of brain

(A–C) H&E–stained brain. (A) Control animals (*repo–Gal4*). (B) *CG11426* knockdown in glia (*repo>CG11426 RNAi*). (C) *CG11426* overexpression in glia (*repo>CG11426*). Increased levels of *CG11426* caused the collapse of the brain cortex. **(A'–C')** Box area from A–C. The arrowhead indicates collapsed cortex. Scale bar, 100 μm.

Chapter 4. Discussion

In the nervous system, glia functions in a variety of ways, and when glia play roles abnormally, it causes various neurological diseases including neurodegeneration. Therefore, study for glia is essential to understand the function of the nervous system. Also, enhanced glia proliferation results in gliomas, the most common human brain tumor (Read et al., 2009; Witte et al., 2009). Therefore, it is necessary to maintain an adequate number of glia. However, surprisingly little is known about the molecular pathways of glia in any organism (Stork et al., 2012). In a previous study, there was an attempt to find a gene with similar expression pattern to Repo in order to find one that affects glia, and the *CG11426* gene was found (Choi, 2017). It was reported that reduced *CG11426* levels in glia led to an increase in glial population in the embryo and the larval eye imaginal disc (Choi, 2017). In this study, the *CG11426* gene was confirmed to be expressed in glia and the ectopic expression of the *CG11426* gene was analyzed. Increased *CG11426* levels decreased glial cell number in the eye imaginal discs, whereas reduced *CG11426* levels increased glial cell number.

CG11426 gene encodes LPP. It was reported that LPA and S1P, which are targets of LPP, are bioactive molecules affecting many cellular processes through GPCRs that induce various responses in vertebrates (Brindley and Pilquil, 2009; Mendelson et al., 2014;

Pyne and Pyne, 2000; Sheng et al., 2015; Tigyi and Parrill, 2003; Yung et al., 2014). For example, LPA and S1P activate mediators such as Rho, Rac, Ras, Akt, and MAPK through GPCRs and these signaling cascades affect cellular processes such as proliferation, migration, apoptosis, morphological changes, and differentiation (Brindley and Pilquil, 2009; Mendelson et al., 2014; Pyne and Pyne, 2000; Sheng et al., 2015; Tigyi and Parrill, 2003; Yung et al., 2014). However, in *Drosophila*, there is a lack of understanding of LPA, S1P, and LPP. Previous studies have reported that LPA and S1P inhibit apoptosis by activating ERK pathway in mammalian cells (Castillo and Teegarden, 2003; Kim et al., 2003; Sui et al., 2015; Wang et al., 2015). We inferred that the new LPP gene, *CG11426,* will also function as such in *Drosophila*. This mechanism of LPA and S1P in *Drosophila* is significant since it was first discovered in *Drosophila*.

Although several studies have identified signaling that regulates glia proliferation such as FGFR, EGFR, JNK, and Hippo signaling, there is insufficient understanding of apoptosis that regulates glial cell number in *Drosophila*. Regulation of the glial population by proliferation usually exhibits a more severe phenotype (Franzdóttir et al., 2009; Hans et al., 2018; Read et al., 2009; Reddy and Irvine, 2011; Witte et al., 2009).The regulation of glial cell number by the *CG11426* gene is regulated by apoptosis, not by proliferation, so less severe phenotypes may have appeared. In the developmental stage, apoptosis is one of the most important mechanisms. Therefore, it is very meaningful to know the regulation of glial

population through apoptosis in the developing eye disc.

Some previous studies described signaling pathways that regulate apoptosis in *Drosophila*; Notch signaling, JNK signaling, and Fat/Hippo signaling pathway induce apoptosis (Arya and White, 2015). On the other hand, RAS/MAPK signaling and PI3K signaling suppress apoptosis (Arya and White, 2015). Glial cell apoptosis by the *CG11426* gene is associated with suppression of ERK signaling. An Increase in dpERK signal was observed in *CG11426* knockdown, and a decrease in dpERK signal was observed in *CG11426* overexpression. It has been reported that ERK inactivates Hid, a cell death protein, by phosphorylation in *Drosophila* (Bergmann et al., 1998). The survival of midline glia has also been reported to depend on such a mechanism (Bergmann et al., 2002). These are consistent with the results that an increase in active ERK and inhibition of apoptosis occurred in *CG11426* knockdown flies and the opposite results in *CG11426* overexpression flies.

In this study, we showed a novel regulation mechanism by which *CG11426* regulates glial cell number in the *Drosophila* eye imaginal disc through apoptosis by inhibiting ERK signaling pathway. *CG11426*, expressed in carpet glia, inactivates extracellular LPA and S1P by dephosphorylation and prevents them from acting as signaling molecules. Therefore, ERK cannot be activated, and Hid cannot be inhibited, resulting in apoptosis (Fig. 15).

Carpet glia, expressing *CG11426* gene, affect the migration of glia in the eye disc (Silies et al., 2007). This study uncovered a new function of carpet glia, which is regulation of the glial

population in the eye imaginal disc. We hypothesized that since LPP encoded by *CG11426* gene normally inactivates extracellular LPA and S1P, the degree of expression of LPP will affect the activation of peripheral LPA and S1P, which in turn will change the surrounding environment and affect the surrounding glial cells. The survival of many cell types depends on trophic mechanisms (Bergmann et al., 2002). We suspect that the *CG11426* gene product normally inhibits the survival of glial cells by suppressing the trophic survival factors, LPA and S1P. Because *CG11426* is expressed in carpet glia and functions on the membrane covering the entire glia region, *CG11426* can affect all of the glia in the eye disc.

Glial cells in the eye disc are essential for appropriate photoreceptor (PR) axon projection (Chotard and Salecker, 2007; Rangarajan et al., 1999). Therefore, an abnormal glial population may cause failure in axon projection. It was reported that the decrease in the number of WG results in abnormalities in the PR axon projection, such as more and larger holes in the lamina (Chang et al., 2018). It was also found in the *CG11426* mutants. Also, the PR axon did not extend widely to the medulla. These results suggest that mutation in *CG11426* gene results in abnormal glial population and consequently failure in axon projection. Also, increased levels of *CG11426* caused the collapse of the brain cortex in the adult fly. Moreover, increased levels of *CG11426* caused smaller brain and eye disc. These indicate that overexpression of *CG11426* results in a reduction of glial population and causes

defects in integrity of brain.

This study demonstrates that *CG11426* normally allows appropriate glial population and that its regulatory mechanism is inducing apoptosis by suppressing ERK signaling (Fig. 15). However, further study is needed to find out which receptors of glial cell lead to ERK signal transduction by the extracellular S1P and LPA. In mammals, S1P and LPA are known to signal through GPCRs, but there is no known receptor of S1P and LPA in *Drosophila*.

Elevated EGFR-Ras signaling is common in gliomas (Hatanpaa et al., 2010; Read et al., 2009; Witte et al., 2009). This study contributes in identifying the suppression mechanism of gliomas. We suggest that *CG11426* suppresses ERK signaling to maintain an appropriate number of glia, which would be a way to prevent gliomas.

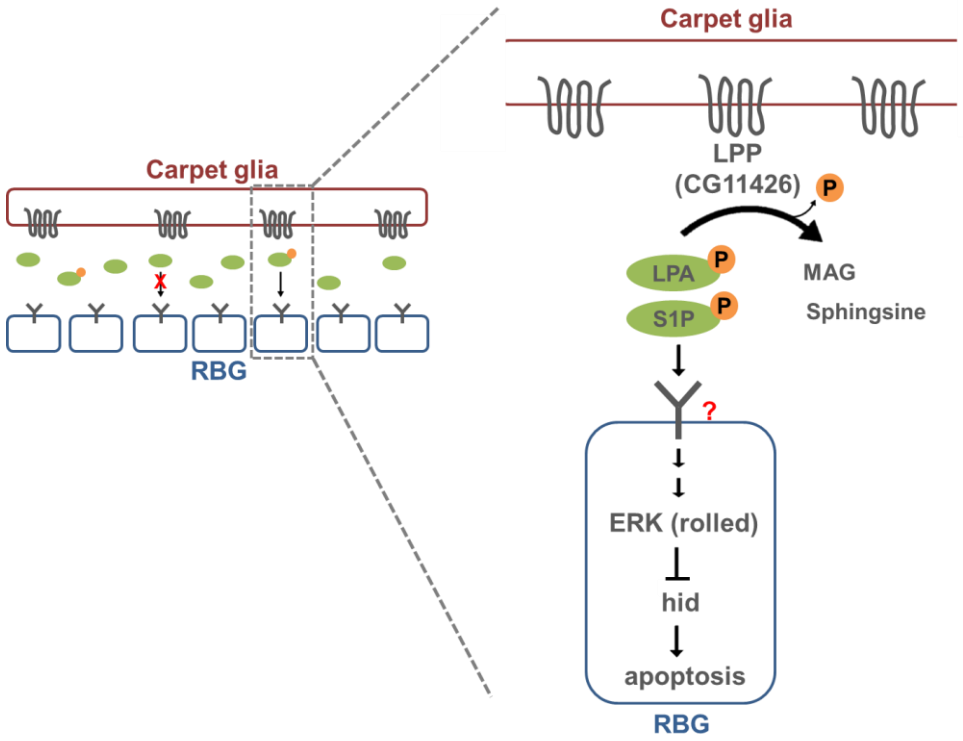

Figure 15. The mechanism by which the *CG11426* regulates glial population

Model: LPPs (*CG11426* gene products), expressed in carpet glia, inactivate extracellular lipid phosphates such as LPA and S1P, bioactive mediators. LPA and S1P cannot activate ERK (in *Drosophila*, rolled) in the adjacent RBG. Therefore, Hid cannot be inhibited, resulting in apoptosis. MAG, monoacylglycerol.

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국문초록

초파리의 눈 성충판에서 *CG11426* 유전자 돌연변이가 신경교세포 수에 미치는 영향

신경계에서 신경교세포는 단순히 신경세포를 보호하는 것 이외에도 다양한 역할을 한다. 신경교세포는 신경 축삭돌기가 뻗어나가는 길을 안내하고, 뇌를 보호하는 장벽을 형성하며, 신경세포에 영양분을 공급하기도 한다. 따라서 신경계를 이해하고자 한다면 신경교세포에 대한 이해가 바탕이 되어야 한다. 초파리의 신경교세포는 사람의 신경교세포와 형태와 기능이 비슷하기 때문에 초파리는 신경교세포를 연구하기에 적합한 모델 동물이다. 이 연구에서는 초파리의 신경교세포에서 발현되는 *CG11426* 유전자를 탐색하였다. *CG11426* 유전자는 기존에 연구되지 않은 인지질 탈인산화효소를 암호화하는 유전자이다. 초파리의 인지질 탈인산화효소라고 알려진 Wunen은 생식세포의 이동과 생존, 정점 접합 형성 및 기관 발달에 영향을 미친다고 알려져 있다. 이 연구에서는 새로운 인지질 탈인산화효소를 암호화하는 *CG11426* 유전자를 탐색하였다. *CG11426* 유전자의 발현을 억제하였더니 신경교세포 수가 늘어났고, 반대로 *CG11426* 유전자의 발현을 증가시켰더니 신경교세포 수가 감소하였다. 또한, *CG11426*

유전자의 발현이 증가하였을 때 유충의 눈-뇌 복합체에서 신경 축삭 말단이 정확한 위치에 도달하지 못했고, 뇌에도 손상이 발견되었다. 이러한 연구의 결과들로 *CG11426* 유전자가 적절한 수의 신경교세포를 유지하도록 조절하며, 그 조절 기작은 ERK signaling 억제에 의한 세포 사멸 때문임을 밝혔다.

주요어: ***Drosophila melanogaster*, 인지질 탈인산화효소, 신경교세포, 세포 사멸, ERK signaling, *CG11426***

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